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INTERFERENCE OF MOLYBDENUM IN THE DETERMINATION OF COPPER

by

R. S. YOUNG and A. GOLLEDGE

OPSOMMING

In teenstelling met die aanbevelings in verskeie naslaanwerke, is dit nie bevredigend om Cu van Mo met Al te skei indien die Cu later volgens die lang-jodied-metode bepaal word nie. Op dieselfde wyse sal Mo steurnis veroorsaak indien Cu met $Na_2S_2O_3$ of Zn verwyder word. In sulke afskeidings moet die kort-jodied-metode, waar Mo nie steurend werk nie, toegepas word.

SUMMARY

Contrary to the recommendations of many reference books, the separation of copper from molybdenum by means of aluminium is not satisfactory for a subsequent long-iodide determination of copper. Separation of copper by sodium thiosulphate or zinc are likewise subject to later interference by molybdenum. If these separations are practised, the short-iodide procedure, in which molybdenum does not interfere, should be employed.

It has been generally recorded that hexavalent molybdenum interferes in the long-iodide procedure for copper[1, 2]. It does not appear to be realized, however, that the method of precipitation by aluminium commonly advocated by authoritative reference books for the isolation of copper from a number of elements, including molybdenum [3, 4, 5], when the latter is present, leads to erroneous results in the long-iodide procedure.

A large number of determinations has been carried out in our laboratory with varying quantities of standard solutions of molybdenum and copper, and concentrations of sulphuric acid for the reduction of copper by aluminium ranging from 5 to 25 per cent by volume. The standard procedure for the precipitation of copper by means of sheet aluminium, and its subsequent iodometric determination, as outlined in the above references and practised by us for many years, was followed.

We do not use hydrogen sulphide water in the separation of copper by aluminium, for we have confirmed the observation of Hillebrand and Lundell that it is quite unnecessary and in some cases may be detrimental by increasing the contaminants.

In no case did the copper found equal the copper added, indicating that in the presence of molybdenum the conventional separation of copper by means of aluminium is not satisfactory. Some typical results selected at random from about a hundred determinations are illustrated here:

Concentration of sulphuric acid by volume	*Weight Mo added g.*	*Weight Cu added g.*	*Weight Cu found g.*
5	nil	0·0643	0·0643
5	0·049	0·0643	0·0606
5	0·49	0·0643	0·0535
10	0·049	0·0640	0·0624
10	0·49	0·0613	0·0580
15	0·049	0·0637	0·0621
20	0·049	0·0640	0·0616
25	0·049	0·0640	0·0617
25	0·49	0·0643	0·0618

A striking feature of these results was the difference in recovery between duplicate samples, which frequently amounted to 5 – 8 per cent. At lower acid concentrations there was a tendency for an increase in molybdenum content to be accompanied by a lower recovery of copper; at higher acid concentrations there was no such relation. For the same quantity of molybdenum there was no clear correlation between concentration of acid and recovery of copper.

The explanation for this interference is not clear. Molybdenum by itself, when boiled under these conditions, is not precipitated on aluminium. The minute quantities which are coprecipitated with copper when the latter is reduced with aluminium must be responsible for this interference in the iodometric determination.

The end point of the titration in all cases was sharp and non-recurring, and the interference cannot be ascribed to this cause. When a solution containing only molybdenum is carried through all the steps of the procedure it does not liberate iodine from potassium iodide in a sodium or ammonium acetate solution.

An attempt was made to substitute zinc for aluminium, in line with the recommendation of a well-known text[6]. The same interference with molybdenum was experienced, three copper samples in 7 per cent sulphuric acid containing 0·049, 0·098 and 0·147 g. molybdenum yielded respectively 91, 97 and 88 per cent recovery of copper.

Sodium thiosulphate is frequently used in the copper industry in place of hydrogen sulphide to precipitate copper. Since sodium thiosulphate precipitates copper as cuprous sulphide instead of the cupric sulphide brought down by hydrogen sulphide, it was thought this modification might have some effect on molybdenum interference in the long-iodide procedure. It was found, however, that molybdenum accompanied copper and interfered to the same extent whether hydrogen sulphide or sodium thiosulphate was used for precipitation of copper.

It appears, therefore, that in the long-iodide procedure for copper the prior separation of the latter from molybdenum cannot be successfully carried out by aluminium. In the presence of molybdenum, copper should be determined by the short-iodide method. The addition of a fluoride in the latter method completely overcomes the interference of molybdenum, probably by the formation of an undissociated molybdenum fluoride complex. The recovery of 0·02 g. copper is complete with the short-iodide procedure in the presence of 0·5 g. molybdenum.

Nkana,
Northern Rhodesia.

Received November 10, 1948.

REFERENCES

[1]Hillebrand, W. F., and Lundell, G. E. F., "Applied Inorganic Analysis", J. Wiley & Sons, 1929, 198.
[2]Scott, W. W., and Furman, N. H., "Standard Methods of Chemical Analysis", 5th Ed., D. Van Nostrand Co., 1938, 368.
[3]Hillebrand, W. F., and Lundell, G. E. F., "Applied Inorganic Analysis", J. Wiley & Sons, 1929, 195.
[4]Scott, W. W., and Furman, N. H., "Standard Methods of Chemical Analysis", 5th Ed., D. Van Nostrand Co., 1938, 369.
[5]Low, A. H., Weinig, A. J., and Schoder, W. P., "Technical Methods of Ore Analysis", 11th Ed., J. Wiley & Sons, 1939, 114.
[6]Lord, N. W., and Demorest, D. J., "Metallurgical Analysis", 5th Ed., J. Wiley & Sons, 1924, 232.

CANE MOLASSES FERMENTATIONS

PART III. AN ACCOUNT OF A SEARCH FOR STRAINS OF ASPERGILLI THAT WILL GIVE GOOD YIELDS OF CITRIC ACID IN STATIONARY CULTURE FROM ALL VARIETIES OF NATAL MOLASSES

by

A. E. OXFORD, H. H. SEPHTON and G. C. A. VAN DER WESTHUIZEN

OPSOMMING

1. Uit 103 tipes van die *A. niger* en *A. wentii* groepe is 23 gevind wat bevredigende gisting van Natalse meulmelasse na sitroensuur in stilstaande kulture kan teweegbring. Baie ander tipes het aansienlike vermoë om dit ook te kan doen.

2. Die optimum konsentrasie van meulmelasse, skyn omtrent 33 persent te wees; 25 persent is definitief te laag en lewer lae opbrengste en 40 persent skyn definitief te hoog te wees vir sommige tipes van *A. niger*.

3. Die beste gisting word verkry teen 'n aanvanklike pH van 5 – 5·5, wat die natuurlike pH van die melasse is, en 'n temperatuur van 20°C. – 28°C.; 'n temperatuur van 32·5°C. is definitief te hoog vir inkubasie.

4. Raffinaderymelasse is ook geskik vir gisting deur tipes wat goeie opbrengste lewer op meulmelasse

5. Skynbaar oefen die anorganiese bestanddele van die meulmelasse nie 'n vertragende uitwerking op die gisting uit nie. Selfs wanneer die anorganiese soutinhoud van die gistingsbodem tot 6 persent verhoog word deur met seewater te meng, verloop die gisting nog steeds bevredigend.

6. *A. niger* ge-isoleer uit grond of beskimmelde plantmateriaal, besit almal 'n aansienlike vermoë om meulmelasse na sitroensuur te fermenteer. Die beste tipes is egter verkry uit buitelandse kultuurversamelings. Die biochemiese eienskappe van sommige van hierdie kulture sal waarskynlik oor 'n lang tydperk nog stabiel bly.

SUMMARY

1. Of 103 strains in the *Aspergillus niger* and *A. wentii* groups, some 23 have been found to ferment Natal mill molasses satisfactorily to citric acid in stationary culture. Many others have an appreciable power of doing this also.

2. The optimum concentration of Natal mill molasses for this fermentation seems to be about 33 per cent; 25 per cent is definitely too low, giving relatively poor yields and 40 per cent seems definitely to be too high a concentration with some strains of *A. niger*.

3. It is best to conduct the fermentation at the natural initial pH of the molasses mash, *viz.*, 5 – 5·5, and at a temperature between 20°C. and 28°C.; 32·5°C. is definitely too high an incubation temperature.

4. Refinery molasses is also satisfactorily fermented by strains which do well on mill molasses.

5. The inorganic constituents of mill molasses do not seem to constitute an inhibitory factor in this fermentation. If the inorganic salt concentration of the medium is increased to as much as 6 per cent by mashing with sea-water, the fermentation still takes place satisfactorily.

6. Strains of *A. niger* isolated from local soil or mouldy plant material all have a fair power of fermenting mill molasses to citric acid. The best strains, however, were obtained from foreign culture collections. Some, but not all, of these would probably remain biochemically stable over long periods in the future.

INTRODUCTION

As stated in Part II*, the only micro-organisms to be considered as producers of citric acid are the black Aspergilli, and strains of *Aspergillus wentii*. With regard to the first-named, Thom and Raper[1] state that "the black Aspergilli are probably more common than any other representatives of the genus. They are world-wide in distribution and occur in and upon the greatest variety of substrata. . . . They are abundant in all soils examined, and . . . it would appear that they are particularly abundant in soils from tropical and sub-tropical areas." Unlike other groups of morphologically similar moulds which happen to be associated with definite fermentation products,

* Oxford, A. E., *S.A. Ind. Chem.*, 1949, **3**, 50.

almost every culture of a black Aspergillus, whether freshly isolated or not, has some power of producing citric acid from sugar under the right conditions. There is therefore an abundance of mycological material available, both in existing cultures in the known culture collections in various parts of the world, and in freshly isolated cultures which can readily be obtained, especially in sub-tropical regions like the Transvaal, from soil and from decaying plant material. Our study includes 92 cultures obtained from culture collections (both public and private) and 7 cultures which we have isolated ourselves on the spot. It should be borne in mind that the black Aspergilli consist of many species besides *A. niger* [*see* Thom and Raper[1]], and as far as fermentative power is concerned the literature gives no warrant for confining our attention to *A. niger* only. Our own results definitely support this conclusion.

The case of *Aspergillus wentii* is rather different in many respects, thus:

(1) It is only recently [Waksman and Karow[2], Karow and Waksman[3]] that strains in this group have received attention as citric acid producers. Their biochemical activities have not received much attention in the past, and are largely unknown. [*See*, however, Birkinshaw and Raistrick[4].]

(2) Morphologically the group is somewhat artificial in comparison with the *A. niger* group, and occupies a position intermediate between that group and other groups which are not noted for their power of producing citric acid, such as the *A. flavus* and *A. ochraceus* groups. According to Thom and Raper[1], the degree of relationship between the species commonly assigned to the *A. wentii* group is open to question.

(3) *A. wentii* is not nearly as common in soil and upon decaying vegetable matter as *A. niger* and related species.

In this study we have included only four strains of *A. wentii*, all obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, and therefore presumably authentic. No attempt has as yet been made to isolate new strains from local natural sources.

At the outset of this work, it was thought probable that those strains which gave good yields of citric acid from Currie's[5] simple medium and under his well-defined conditions, with refined sugar as a sole source of carbon in the medium, would also do best on molasses as citric acid producers [*cf.* Chatterjee[6]]. This soon proved to be so far from the case that the routine use of Currie's simple liquid medium was discontinued, and new strains, as received, were tested directly upon the crudest mill molasses, of the highest ash content, instead. There are at least three kinds of molasses produced in Natal, *viz.*, ordinary mill molasses; Natal Estates molasses (formed as an end-product in a double-carbonatation process, which, being different in many particulars from the practice in most of the other mills in Natal, gives a molasses the ash of which is of rather unusual composition, containing relatively little sulphate); and refinery molasses, which is naturally less contaminated with organic nitrogenous substances than mill molasses. Our study has unfortunately not included double carbonatation molasses, but as far as our experience goes, the behaviour of a given strain upon the crudest mill molasses does give a fair indication of its performance upon the other varieties of molasses at our disposal. In deciding, therefore, which of the black Aspergilli in our collection were worth trying out under special conditions to be described in later communications in this series particularly, we have been guided by the results obtained with mill molasses described in this section.

EXPERIMENTAL

I. Strains used

(*a*) *A. niger*. The S.A. National Chemical Research Laboratory culture collection numbers for the strains used are as follows:—

220 – 235, 288 – 290, 292 – 293, 297, 307 – 312, 372 – 293 A & B, 396 – 411, 449, 454 – 5, 459, 469 – 472, 478 – 493.

(*b*) *Black Aspergilli*.

A. carbonarius ..	412, 473.
A. cinnamomeus ..	416, 474.
A. fuscus	415.
A. luchuensis ..	418, 419, 475.
A. niger-citricus ..	304, 420.
A. pulverulentus ..	413, 476.
A. violaceo-fuscus ..	417.

(*c*) *A. wentii*. Nos. 238 – 241.

The above include strains from the American Type Culture Collection (A.T.C.C.), the National Research Council of Canada (N.R.C.), the Centraalbureau voor Schimmelcultures (Holland) (C.B.S.), the Northern Regional Research Laboratory (U.S.A.) (N.R.R.L.), the Timber Research Laboratory of the Chamber of Mines (Johannesburg) (T.R.L.J.), the National Collection of Type Cultures (Lister Institute, England) (N.C.T.C.), Prof. Simonart (University of Louvain, Belgium) (Simonart), the London School of Hygiene and Tropical Medicine (L.S.H.T.M.), as well as a number of strains from local sources.

II. Maintenance of cultures

All the above strains were maintained on slopes of prune agar, made according to the directions of Smith[7]. When good growth with sporulation had occurred at room temperature (say, in a week) the slopes were transferred to a refrigerator and kept there until required. Usually 9 – 12 months were allowed to elapse between routine sub-culturings for maintenance of the collection.

With reference to the maintenance of the L.S.H.T.M. strains before receipt by us, Mr. G. Smith very kindly wrote as follows: "As far as I know the older strains were kept on glucose-Czapek agar before I had them in 1931. I never use this medium for stock cultures, but transfer more or less alternately on to Czapek with 3 per cent sucrose, wort agar and potato agar."

Our practice has been to sub-culture on to glucose—or sucrose—Czapek agar slopes at pH 5 [made also according to the directions of Smith[7]] in order to obtain inocula for the liquid media used for fermentation studies. The reason for so doing is that moulds tend to grow more luxuriantly on prune agar than on Czapek agar, with production of much aerial mycelium. Since it is desired to have the minimum growth on the liquid medium, consistent with a good fermentation, it was considered good practice to take inocula from an abundantly sporing culture with only sparse aerial mycelium, *i.e.*, from the growth on glucose—or sucrose—Czapek agar in preference to prune agar. As indicated by Mr. G. Smith it is not good practice to maintain cultures indefinitely on a poor medium like glucose—or sucrose—Czapek agar. A risk is run thereby of losing the culture through premature death or through an undesirable mutation with loss of biochemical characteristics.

III. Fermentation media

The following have been used:—

Currie S. Best refined sugar, 12·5 per cent; NH_4NO_3, 0·2 per cent; $MgSO_4.7H_2O$, 0·02 per cent; KH_2PO_4, 0·075 per cent; HCl (1 – 1·5 ml. of 5N–HCl per litre) to bring to pH 2 – 2·6.

Currie G. As Currie S, save that brown not fully-refined "Government" sugar was used in place of best refined sugar.

Currie M. As Currie S, save that mill molasses (ca. 25 per cent) was used in place of best refined sugar and much more HCl (30 ml. of 0·5N–HCl per litre) was needed to reduce the pH to 3. *The molasses* used in this and the following media had the following composition: sucrose, 36·5 per cent; invert sugar, 11·7 per cent; total fermentable sugar, 48·2 per cent; ash by ignition at 750°C., 11·4 per cent. The ash had the following composition (calculated as percentage of original molasses): SiO_2 + insoluble in acid, 0·2 per cent; $Fe_2O_3 + Al_2O_3$, 0·3 per cent; CaO, 1·5 per cent; MgO, 1·1 per cent; SO_3, 2·5 per cent; Cl, 2·1 per cent; CO_3, trace; PO_4, nil; unaccounted for (K_2O and Na_2O chiefly), 3·7 per cent.

The molasses contains enough calcium oxide to precipitate the greater part but not all of the sulphate as $CaSO_4$ when the medium is made up, leaving only 0·4 per cent SO_3 available for the mould. The "effective" ash content of the molasses, *i.e.*, the concentration of soluble inorganic salts in it is thus only 7·6 per cent. Since molasses contains no phosphate or nitrate, it was thought necessary to add these salts to molasses media.

*M*25. As Currie M, save that no HCl was added to lower the pH. The pH of the medium was 5·5 and its fermentable sugar content about 12 per cent

*M*22*G*. As M25, save that it contains only 22 per cent of molasses and has 9·5 per cent of Government sugar in addition. The fermentable sugar content is thus about 19 per cent.

*M*33. As M25, save that the concentration of molasses was 33 per cent, corresponding to a fermentable sugar content of about 15 per cent (found: sucrose, 10·8 per cent; invert sugar, 3·83 per cent). None of the molasses media was filtered before use.

IV. Fermentation conditions

In general, the fermentations were carried out in 12 – 15 ml. quantities in plugged boiling tubes (15 × 2·5 cm.). Sometimes small conical flasks (100, 250 or 500 ml.) were used, filled about one-third full with medium. All media were sterilized by steaming for 1 hour on each of two successive days. Incubation was usually at room temperature (20°C. – 27°C.) for approximately 2 – 3 weeks, unless otherwise stated. In this time an uninoculated tube containing 15 ml. of medium, lost 4 ml. on the average through evaporation. A heavy inoculum was used whenever possible to ensure quick formation of a complete felt. In experiments with strains 311 and 220 on Currie S, the acidities after 15 and after 24 days' incubation were identical, and neither culture contained oxalic acid. It was concluded, therefore, that there was little danger of loss of citric acid by over-incubation, if the cultures were worked up after 3 weeks.

V. Analysis of fermentation liquids

The felt was drawn up to the top of the tube and before removal was squeezed with a spatula, the tube being in an inclined position so that the expressed liquid could

run back. The total metabolic liquid was then filtered without suction through a fluted No. 1 paper. Titratable acidity was determined by titration using 0·04 N–NaOH and phenolphthalein as indicator. Dividing the net titre by 4 gave an approximate value for fermentation acids calculated as citric. With molasses media the procedure was to dilute 1·0 ml. with water (50 ml.) to lessen the colour and then to add rather much phenolphthalein (1 or 2 ml. of 0·2 per cent solution in aqueous alcohol). The end point was taken to be the first unequivocal appearance of a reddish-brown tinge in the yellow liquid. Citric and oxalic acids were occasionally determined by the methods described in Part IV*.

* This journal.

RESULTS

1. Comparison of citric acid production from Currie S and Currie M media at pH 2 · 3

At pH 2 – 3, Currie M was so unsuitable a medium for many strains of *A. niger* that no detectable amount of citric acid was produced in it. Most of the strains listed under (I) above were compared on this medium at pH 2 – 3, but only Nos. 225 and 389 gave any appreciable yields of citric acid (0·85 and 1·05 per cent of citric acid in the final media respectively). Under the same conditions but using Currie S medium, reasonable yields of citric acid (1·8 to 5·65 per cent in the final medium) were obtained with a number of strains.

2. Influence of pH on citric acid production from molasses

From the above experiments it was clear that the initial pH of the medium was of considerable importance with molasses as substrate. A number of strains was therefore compared on Currie M (initial pH, 3·5) and M25 (initial pH, 5·5) media. It was found that most of the moulds tested made more citric acid starting at the natural pH of the molasses (pH 5·5) than at pH 3·5.

3. Influence of sugar concentration on citric acid production in molasses media at pH 5·5

Since it is a well-known fact that the percentage yield of citric acid from sugar in the fermentation of Currie's simple medium is greater with increasing concentration of sugar, the optimum being 15 per cent sucrose or more, the effect of increasing the sugar concentrate of the molasses medium by addition of sugar ("Government Grade") was explored. Typical results are listed in Table I, which also shows that better results are obtained with high initial sugar concentration if the initial pH is 5·5 and not 3·5.

Notes on Table I

(i) The main acidic metabolic product was undoubtedly citric acid, and in no instance was an appreciable amount of oxalic acid detected. This was proved by combining the metabolic liquids of high, medium and low acidities separately, and preparing calcium citrate from each by neutralization, addition of excess $CaCl_2$ solution, and heating to b.p. until separation of the calcium salt took place. After standing for a day, the calcium citrate was collected, well washed and air-dried.

Results:

62 ml. of high acidity (16·0 ml. 0·04N. per ml.) yielded 3·045 g. calcium citrate.

TABLE I

Effect of sugar concentration and initial pH on the production of citric acid from molasses media.

Medium	*Strain*	*Per cent citric acid in final liquor*	*Strain*	*Per cent citric acid in final liquor*	*Strain*	*Per cent citric acid in final liquor*
Currie S	220	5·3	375	1·3	311	5·65
Currie M	220	0·0	375	0·5	311	0·0
M25	220	—	375	—	311	—
M22G	220	5·75	375	4·25	311	4·8
Currie S	225	1·8	383	—	373	—
Currie M	225	0·85	383	1·45	373	0·2
M25	225	—	383	1·0	373	1·3
M22G	225	3·75	383	2·4	373	3·6
Currie S	238	—	386	3·1	293	3·2
Currie M	238	No growth	386	0·4	293	0·4
M25	238	1·2	386	—	293	—
M22G	238	0·5	386	2·0	293	4·7
Currie M	239	No growth	387	—		
M25	239	1·2	387	—		
M22G	239	1·4, 1·5	387	5·15		
Currie M	289	0·0	389	1·05		
M25	289	0·4	389	—		
M22G	289	2·9	389	2·25	397	1·5
M22G (pH3) ..	384	1·0	393A	0·3	392	0·7
M22G (pH 5·5) ..	384	2·1	393	0·8	392	2·4
M22G (pH 3) ..	469	1·05	471	0·7	472	0·95
M22G (pH 5·5) ..	469	3·0	471	4·7	472	4·35
M22G (pH 3) ..	454	0·65	455	1·55	470	2·2
M22G (pH 5·5) ..	454	2·7	455	3·35	470	2·0

75 ml. of intermediate acidity (10·4 ml. 0·04N. per ml.) yielded 1·40 g. calcium citrate, of 82 per cent purity (by colorimetric method).

100 ml. of low acidity (7 ml. 0·04N. per ml.) yielded 1·368 g. calcium citrate.

47 ml. of low acidity from fermentations starting at pH 3 (3·9 ml. 0·04N. per ml.) yielded 0·45 g. calcium citrate, of 78 per cent purity (by colorimetric method).

(ii) As might be expected, duplicates did not always show close agreement. The reason for this was later found to lie partly in the fact that no special precautions were taken to prevent the fermentation tubes from being moved during incubation. It is of the utmost importance that the surface felt of mould growth should not be disturbed. A slight jerk is often sufficient to cause a part or the whole to become waterlogged and even to sink to the bottom of the liquid.

(iii) It would seem that the behaviour of a given strain on Currie S gives no certain indication of the behaviour to be expected on M22G. Thus, with seven strains the order of acid production on Currie S was, 311 >220 >293 >386 >389 >225 >375, whereas the order on M22G was 220 >311 >293 >375

>225 >389 >386. Strains 375 and 225 particularly showed a much better performance on the molasses medium, whereas 386 did much better on refined sugar.

4. Effect of the non-sugar components of molasses on the citric acid fermentation

In view of the unexpected difference between certain results upon Currie S and upon M22G media, the effect of replacing various proportions of the sucrose in Currie S with molasses containing an equivalent amount of fermentable sugar was tried. The strains used for this test, Nos. 293 and 389, were of moderate fermentative power. Table II shows that whereas replacement of sucrose by molasses had little effect upon 389, it has a marked but irregular effect upon 293. This confirms the conclusion reached above that no certain deductions as to behaviour on a molasses medium can be drawn from experiments using a sucrose medium.

Further, it could be demonstrated that even the small amount of molasses contaminating the "Government grade" of sugar is liable to affect the citric acid fermentation to an appreciable, if rather erratic, degree. The experiments in this connection were carried out at 32·5°C. and all gave lower yields than previous experiments at lower temperatures.

TABLE II(*a*)

Effect of replacement of sucrose of Currie S medium with molasses of equivalent sugar content.

Concentration in medium of		*Concentration of citric acid produced from*	
Sucrose per cent	*Molasses per cent*	*Strain No.* 293	*Strain No.* 389
0	25	0·5	1·1
2·5	20	1·2	1·3
4·0	17	1·1	1·2
6·0	13	1·0	1·0
7·5	10	1·5	1·15
9·0	7	1·7	1·05
11·0	3	1·5	1·15
12·5	0	1·05	1·25

TABLE II(*b*)

Comparison of citric acid yields from sucrose and "Government Grade" sugar at 32·5°C.

Strain	220	293	225	311	375	386	389
Per cent citric acid in liquor from Currie S ..	1·3	1·2 0·9	2·0	1·15 1·35	1·2	0·9	0·55 0·7
Per cent citric acid in liquor from Currie G ..	0·4	1·7 1·3	2·5	0·7 0·5	0·9	0·4	1·15 1·2

5. Fermentation of 33 per cent molasses medium at its natural pH

Since the foregoing experiments had shown clearly that the effect of the non-sugar components of molasses upon the fermentation varied greatly from strain to strain,

it was condluded that it would be preferable to carry out the routine testing upon a molasses medium of high sugar content. Table III therefore summarizes the results obtained in a series of such tests and includes all the good citric acid strains in the National Chemical Research Laboratory collection as well as many others. In a few cases 40 per cent molasses medium was used for comparison purposes.

TABLE III

Fermentation of Medium M33 by numerous black aspergilli and strains of A. wentii.

Strain	*Per cent citric acid in medium*	*Remarks*	*Strain*	*Per cent citric acid in medium*	*Remarks*
220	4·3	–/4/48	221	3·85	
220	3·55	–/5/48	224	4·05	
220	4·25	–/8/48	224	1·6	40 per cent molasses medium
225	4·55		289	3·2	
239	1·4		289	2·9	40 per cent molasses medium
293	5·4	–/4/48	307	1·65	
293	5·0	–/5/48	308	0·25	
293	4·05	–/5/48	310	3·65	
311	4·85	–/4/48	312	1·4	–/4/48
311	3·1	–/5/48	312	0·6	–/7/48
311	3·4	–/7/48	373	3·65	–/4/48
375	6·5	Separate cultures	373	4·05	–/5/48
375	4·55		378	1·7	
375	4·85		383	4·55	
386	5·45	From molasses agar slope	387	10·25	–/5/48
386	2·3	From Currie S agar slope	387	10·7	–/5/48
386	3·9	From Currie S agar slope	387	4·6	–/7/48
389	4·25		393B	3·5	40 per cent molasses medium
389	3·0	40 per cent molasses medium	396	3·0	
401	2·3		402	4·55	
403	2·5		402	2·5	do.
404	7·45	–/4/48	413	5·75	
404	9·15	–/5/48	413	5·5	do.
469	4·0		413	7·4	
470	4·2		459	4·65	
471	4·6		455	3·7	
472	5·05		478	2·5	do.
473	0·15		479	1·05	do.
474	0·9		480	2·6	do.
475	0·3		481	0·95	do.
476	3·45		482	6·2	do.
485	6·2		483	3·2	do.
486	2·05		484	2·3	do.
487	1·55		484	2·1	
488	2·5		491	1·5	do.
489	3·05		492	3·5	do.
490	2·55		493	1·45	do.

It will be noted that it was of no advantage to increase the molasses content of the medium from 33 per cent to 40 per cent.

If a yield of 25 per cent or more of the fermentable sugar supplied is regarded as promising, then the following 23 strains are worthy of further consideration, since the fermentation liquids to which they gave rise contained more than 3·6 per cent of titratable acidity as citric acid: 220 (A.T.C.C. 1015, U.S.D.A. Strain T.C. 167), 221 (A.T.C.C. 6276, Strain 244–5373.16), 224 (A.T.C.C. 9142, N.R.R.L. Strain 599), 225 (A.T.C.C. 9642, W. H. Weston), 293 (locally derived), 311 (A.T.C.C. 6276 via Univ. Witwatersrand), 373 (Simonart 1686), 375 (Simonart 1688), 383 (Simonart 1703), 386 (Simonart 1707), 387 (Simonart 1708), 389 (Simonart 1711), 402 (L.S.H.T.M. BB1, B.C.I.R.A. 1926), 404 (L.S.H.T.M. A79, W. H. Peterson, Wisconsin), 413 (L.S.H.T.M. Ac.12), 469–472 (local soil), 459 (local soil), 455 (diseased peanut), 482 (Simonart 1732), 485 (Simonart 1735). Of these, 387, 404, 413, 482 and 485 are the best, giving yields of 40 per cent or more.

6. The fermentation of other varieties of Natal molasses by good citric acid strains

Two refinery molasses and two other mill molasses were compared side by side with the mill molasses used heretofore, the medium being M33 in each instance. Three of the best strains, as indicated in Table III, were used, *viz.*, Nos. 387, 404 and 413; cultures were in boiling tubes (15 ml. in each) for the first two and in Erlenmeyer flasks (75 ml. in a 250 ml. flask) for strain 413. The results are summarized in Table IV. The incubation period was 15 days at 25°C. – 26°C.

TABLE IV

Fermentation of Medium M33 prepared from various specimens of molasses.

Description of molasses	*Ash content*	*Sucrose per cent*	*Reducing sugar per cent*	*Total sugar per cent*	*pH of medium*	*Titratable acidities calculated as citric acid per cent*			*Citric acid determined colorimetrically per cent*		
						387	404	413	387	404	413
Refinery, 1947 ..	4·8	39·2	15·5	54·7	5·4	4·45	8·4	6·0	—	—	6·0
Refinery, 1948 ..	3·8	34·8	22·0	56·8	4·9	3·1	8·55*	7·9	—	10·2	6·7
Mill: Amatikulu, 1948	5·3	31·6	12·5	44·1	6·0	3·5	4·8	6·2	—	—	5·5
Mill: Felixton, 1948	4·7	30·8	13·4	44·2	5·7	3·4	6·3	8·45	4·0	7·4	8·3
Mill: N.C.P. as used in previous work	11·4	36·5	11·7	48·2	5·2	4·1	4·15	6·5†	—	5·0	6·8

* Contains 0·09 per cent oxalic acid.
† Contains 0·19 per cent oxalic acid.

7. The fermentation of molasses media containing an abnormally high content of inorganic salts

It was desired to find out if the factor limiting the concentration of molasses in a fermentable medium was too high a concentration of inorganic salts or too high a concentration of organic compounds other than sugars. Fermentations were therefore

carried out in a 33 per cent molasses medium with sea-water (SW) and also with equal parts of sea- and fresh-water (1/2 SW) as mashing fluids. The concentration of inorganic salts in the medium made with sea-water is of the order of 6 per cent, rather less than half of which is due to the molasses. The effect of individual inorganic salts is discussed in Part V‡. The only inorganic substances added to the sea-water mashes in the experiments summarized in Table V were NH_4NO_3 (0·2 per cent) and H_3PO_4 (0·023 per cent). These are essential for the growth of the mould and are absent from both molasses and sea-water.

TABLE V

Fermentation of Medium M33 made up with sea-water (SW) and with a mixture (1/2SW) of equal parts of sea- and fresh-water: incubation period, 18 *days.*

Strain	220	383	387	387	396	404	413	482	485
Citric acid formed§ in 1/2SW medium ..	4·0	—	4·95	5·0	2·4	7·5	5·6	3·6	3·9
Citric acid formed§ in SW medium	—	2·9	4·65	5·25	1·9	7·05	5·6	3·5	4·4

§ Citric acid determined by colorimetric method.
‡ This journal, page 95.

Note on Table V

A specimen of air-dried calcium citrate was prepared from the combined filtered fermentation liquids and was found to be of 73 per cent purity by the colorimetric method.

[*N.B.* The best preparation of air-dried calcium citrate (prepared from a pure sucrose fermentation liquid) so far obtained was of 89 per cent purity.]

DISCUSSION

Comparising the results in Tables I – V it will be seen that, within limits, Natal cane molasses, of different grades and origins, is on the whole as good a substrate for citric acid fermentation as best refined sugar, if yield alone is considered. It would seem inadvisable to include much more than 33 per cent of molasses in the medium—whereas the sucrose content of a refined sugar medium can be safely increased to as much as 22·5 per cent [Bernhauer, Iglauer and Knobloch[8]]—but no doubt each specimen of molasses would have to be considered on its merits in this respect. Best results were obtained with a molasses of abnormally high ash content. The chief factor which reduces yields with very high concentrations of molasses however, is not, the inorganic salt content, since good fermentations were obtained with molasses of high ash content mashed with sea-water. It is advisable, in fact, to work with the highest possible concentration of molasses, otherwise yields will be low.

It is a little difficult to reconcile these conclusions with American experience of the citric fermentation of cane molasses. Perlman, Kita and Peterson[9], and Perlman, Dorrell and Johnson[10], obtained low yields with unpurified cane molasses and attributed these low yields to the inhibitory effect of unidentified inorganic constituents. They recommended the removal of cations and addition of iron salts to the medium. We have, of course, worked with many more strains of black Aspergilli than the American chemists, and thus have perhaps obtained a better overall view of the molasses fermentation, but it is, of course, possible that Cuban or Porto Rican blackstrap does

in fact contain inhibitory cations which are not so much in evidence in Natal Mill Molasses. Only a detailed chemical analysis of the respective molasses can settle this point. One very simple explanation is that the inhibitory cation may be simply calcium which, present in excess, would precipitate all the sulphate from the medium.

There is good reason to believe that a strain of *A. niger* which gives a good yield of citric acid from one specimen of mill molasses, will give good yields from other specimens of mill molasses of different origins, and from refinery molasses also. The best strains so far encountered have all been obtained from culture collections abroad, but strains recently isolated from local soils and infected plant material have all shown a fair power of producing citric acid from sugar. A search for other such strains, therefore, is well worthwhile and is in progress. Another worthwhile investigation arising out of this work would be to find the best solid medium on which to grow the inoculum for the liquid fermentation proper. It is by no means certain that sugar-Czapek-agar, hitherto used for the most part, gives the best results. This point is taken up again in a later communication in this series.

With reference to the stock cultures themselves, the difficulty of maintaining their fermentative powers over a period of years is apparent from the considerations which follow. The following groups of stock cultures, in which the individual cultures were obtained from different sources, yet had the same ultimate origin: thus, 221, 310, 311 and 312 are all derived from A.T.C.C. 6276; 308 and 396 are both derived from L.S.H.T.M. Ac. 1 (fermentative powers reported on in 1931) and 309, 397 are both derived from L.S.H.T.M. Ac. 2 (fermentative powers likewise reported on in 1931). Nevertheless, reference to Table III shows that in the first group 221, 310 and 311 are far superior to 312; in the second group 396 is far superior to 308 and retains the power of producing non-volatile acids it had 17 years ago [Birkinshaw *et al.*[11]], whereas neither 309 nor 397 (the former has been tested on Currie S, the latter appears in Table I) have retained the fermentative power reported by Birkinshaw *et al.*[11].

It appears that some strains are inherently stable with respect to biochemical characteristics, while others are not. Thus, 221, reported upon six years ago, is still a good citric acid producer, likewise Nos. 396, 412 and 413, all reported upon 17 years ago* by Birkinshaw *et al.* Of these, No. 314 (*A. pulverulentus*) is outstanding and if maintained as it has been in the past, would doubtless retain its fermentative power indefinitely. It is interesting that the other strain of *A. pulverulentus* in our collection (No. 476 obtained from the Centraalbureau voor Schimmelcultures and doubtless an old stock culture) had also a fair power of producing citric acid from molasses (*see* Table III). Of the four strains of *A. wentii*, only 239 had an appreciable but not outstanding power of producing acids from sugar. The biochemical stability, or otherwise, of strains recently isolated from soil and mouldy plant material will be reported upon more fully in a later communication in this series.

In conclusion, it should be mentioned that very little attention has here been paid to the nature of the non-volatile acids produced, apart from showing that the chief constituent is in reality citric acid. The important question of concurrent oxalic acid production in this fermentation is considered in detail in Part V of this series. Gluconic acid may also be formed and its presence doubtless largely accounts for the discrepancy in those instances where the combined citric and oxalic percentages fall considerably short of the gross percentage of citric acid calculated from the titratable acidity of

* Actually, the work reported in 1931 was carried out at least five years earlier (private communication from Professor Raistrick).

the fermentation liquid only. Very little attention has likewise been paid to the compounds in the mould mycelium. If the data furnished by Prill, Wenck and Peterson[12] for another Aspergillus also apply to *A. niger*, then the conditions in the molasses fermentation are favourable to the deposition of fat in the mycelium.

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National Chemical Research Laboratory,
South African Council for Scientific and Industrial Research,
Pretoria.

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REFERENCES

[1] Thom, C., and Raper, K. B., "A Manual of the Aspergilli", The Williams & Wilkins Co., Baltimore, 1945.
[2] Waksman, S. A., and Karow, E. O., U.S.P. 2394031 (*Chem. Abstr.*, 1946, **40**, 2265).
[3] Karow, E. O., and Waksman, S. A., *Ind. Eng. Chem.*, 1947, **39**, 821.
[4] Birkinshaw, J. H., and Raistrick, H., *Phil. Trans. Roy. Soc.*, 1931, **220B**, 331.
[5] Currie, J. N., *J. Biol. Chem.*, 1917, **31**, 15.
[6] Chatterjee, N. P., *J. Indian Chem. Soc.* (Ind. & News Ed.), 1942, **5**, 201 (*Chem. Abstr.*, 1943, **37**, 6813)
[7] Smith, G., "An Introduction to Industrial Mycology", 3rd Ed., E. Arnold & Co. Ltd., London, 1946.
[8] Bernhauer, K., Iglauer, A., and Knobloch, H., *Biochem. Z.*, 1941, **307**, 298.
[9] Perlman, D., Kita, D. A., and Peterson, W. H., *Arch. Biochem.*, 1946, **11**, 123.
[10] Perlman, D., Dorrell, W. W., and Johnson, M. J., *Arch. Biochem.*, 1946, **11**, 131.
[11] Birkinshaw, J. H., Charles, J. H. V., Raistrick, H., and Stoyle, J. A. R., *Phil. Trans. Roy. Soc.*, 1931, **220B**, 27.
[12] Prill, E. A., Wenck, P. R., and Peterson, W. H., *Biochem. J.*, 1935, **29**, 21.

CANE MOLASSES FERMENTATIONS

PART IV. THE DETERMINATION OF CITRIC ACID IN MOLASSES FERMENTATION MEDIA

by

D. J. S. GRAY

OPSOMMING

'n Kolorimetriese metode vir die bepaling van sitroensuur in oplossings van suikerrietmelasse, gefermenteer deur *Aspergillus niger*, word beskryf. Hierdie metode is 'n wysiging van die metode wat afhang van die omsetting van sitroensuur na pentabromoaseton en die kleurreaksie wat hierdie verbinding met natriumsulfied ondergaan. Die reeds bestaande metodes het onbevredigend geblyk, waarskynlik weens onvolledige omsetting van sitroensuur na pentabromoaseton. Die reaksie is ondersoek en 'n kwantitatiewe metode word voorgestel.

Die invloed van steurende stowwe word bespreek.

SUMMARY

A colorimetric method for the determination of citric acid in cane molasses solutions, which have been fermented by *Aspergillus niger*, is described. This method is a modification of the method depending on the conversion of citric acid to pentabromoacetone, and the colour reaction between this substance and sodium sulphide. Existing techniques were found unsatisfactory when applied, and this appeared to be due to the inconsistency of the conversion of citric acid to pentabromoacetone. This reaction has been investigated and a quantitative procedure is proposed.

The influence of interfering substances is discussed.

In studying the fermentation of cane molasses solutions to citric acid with *Aspergillus niger*, it was necessary to have a rapid and reasonably accurate method for the determination of the amount of citric acid produced.

The reaction which has found the most important application to the analysis of citric acid was described by Stahre[1] in 1897. He demonstrated that when citric acid was oxidized with potassium permanganate in the presence of bromine water, pentabromoacetone was formed. The course of the reaction was studied by Wohlk[2], who confirmed that the final product was pentabromoacetone. This reaction has been successfully applied to the gravimetric determination of citric acid.

Pucher, Vickery and Leavenworth[3] have adapted the pentabromoacetone reaction to a volumetric technique. The oxidation to pentabromoacetone is accomplished in a solution which is normal with respect to sulphuric acid, by means of potassium bromide and potassium permanganate. The pentabromoacetone formed is dehalogenated with sodium sulphide by the method described by Kretov, Panchenko and Savich[4], and the sodium sulphide solution is decomposed and its bromine content determined by argentometric titration.

Pucher, Sherman and Vickery[5] have developed a colorimetric method based on the yellow colour observed to be produced when pentabromoacetone is dehalogenated with sodium sulphide, and their technique has been modified by Perlman, Lardy and Johnson[6]. The two methods, last mentioned, proved unsatisfactory when applied by the author to the analysis of molasses fermentation media, and it was found impossible to prepare calibration curves for the Spekker absorptiometer using the recommended technique. Since an examination of the colour produced by the reaction of solutions of pentabromoacetone with sodium sulphide showed that its intensity was related to the concentration of pentabromoacetone in the solution, it appeared

that oxidation of citric acid to pentabromoacetone was not quantitative under the experimental conditions recommended.

Krebs and Eggleston[7] have noted that the amount of pentabromoacetone produced is dependent on the concentration of acid in the solution. This finding has been confirmed by Goldberg and Bernheim[8], who state that the degree of acidity of the solution probably determines the amounts of tri-, tetra-, penta- and hexabromoacetone formed. They have isolated tri- and tetrabromoacetone in solutions where the reaction has taken place under conditions of low acidity, and hexabromoacetone where the acidity is very high. Their findings indicate that pentabromoacetone will be produced quantitatively when the concentration of sulphuric acid in the solution is approximately 6 N.

In their examination of the principles underlying the conversion to pentabromoacetone Goldberg and Bernheim found that the presence of manganous salt was essential to the reaction. They state that the manganous salt necessary for the reaction was probably unwittingly supplied by reduction of potassium permanganate by potassium bromide in the techniques referred to above, and recommend the addition of manganous sulphate to the reaction solution.

Taussky and Shorr[9] have adapted the principles outlined by Goldberg and Bernheim to a micro-colorimetric method for the estimation of citric acid. The colour reaction used is between pentabromoacetone and sodium iodide.

The method which has been developed for the determination of the citric acid content of molasses fermentation media is an adaptation of the Taussky and Shorr technique. As their method is extremely sensitive and is designed for micro-quantities of citric acid, the rather less sensitive sodium sulphide reaction has been substituted for the sodium iodide colour reaction.

Reagents Required

1. *Sulphuric acid*, 27 N. 750 ml. concentrated sulphuric acid added to 250 ml. distilled water. Cool and dilute to 1,000 ml.
2. *Bromate-bromide solution.* 10·5 g. $KBrO_3$ and 37·2 g. KBr dissolved and diluted to 500 ml.
3. *Hydrazine solution.* 32·5 g. $N_2H_4.H_2SO_4$ dissolved with the aid of 49 ml. of 20 per cent NaOH and diluted to 100 ml.
4. *Potassium permanganate*, 5 per cent. 50 g. $KMnO_4$ dissolved in 1,000 ml. water.
5. *Manganese sulphate*, 30 per cent. 300 g. $MnSO_4.H_2O$ dissolved in 1,000 ml. water.
6. *n-Hexane.* Commercial grade was used by the author.
7. *Sodium sulphide.* 10 g. $Na_2S.9H_2O$ dissolved in 250 ml. water.
8. *Glycerol*, 50 per cent. 250 ml. glycerol diluted to 500 ml. with distilled water.
9. *Citric acid standard solution.* 5 g. pure anhydrous citric acid dissolved in 1,000 ml. of N. sulphuric acid. 1 ml. of the resulting solution is equivalent to 5 mg. citric acid. This solution may be diluted as required.

Method

10 ml. of a solution containing 2·0 mg. or less citric acid are pipetted into a 60 ml. glass-stoppered bottle. 5 ml. 27 N. sulphuric acid is added and 2·5 ml. bromate-bromide solution. The bottle is kept in a bath which is maintained between 15 and 20°C. When the solution has attained the temperature of the bath, the bottle is removed, and 1 ml. manganese sulphate solution and 2 ml. potassium permanganate are added. The bottle is shaken to mix its contents and replaced in the water-bath for 30 minutes.

At the end of this period the bottle is removed from the bath and all traces of manganese dioxide and bromine are removed by very careful dropwise addition of hydrazine solution. Care must be taken to avoid an excess otherwise the results obtained will be unreliable. 10 ml. *n*-hexane are added to the solution, and the bottle is stoppered and shaken vigorously for half to one minute. The contents are transferred to a 100 ml. separating funnel, and the hexane layer is allowed to separate. The aqueous layer is returned to the bottle and shaken with a further 5 ml. hexane. The hexane layer from the first separation is transferred to a 50 ml. separating funnel for washing, and the contents of the bottle are poured into the 100 ml. funnel. The hexane layer is allowed to separate, the aqueous layer is run off and discarded, and the hexane layer is combined with the first extract. The bottle and the separating funnel are rinsed with distilled water and these washings are used to wash the hexane extract. The hexane extract is washed twice more with distilled water and is then transferred to a clean, dry test-tube. The washings are discarded. 10 ml. of the extract is shaken with 5 ml. of a mixture of equal volumes of the sodium sulphide and glycerol solutions, in a 50 ml. separating funnel. The lower aqueous layer, which is now yellow in colour, is run into a centrifuge tube, and the hexane extract is shaken with a further 5 ml. of the sodium sulphide-glycerol solution. This extract is combined with that first obtained. The solution is centrifuged to remove any remaining hexane and the developed colour is measured in an absorptiometer.

For the work described a Spekker absorptiometer was used, with Chance's glass filters, type O.B.1.

DISCUSSION

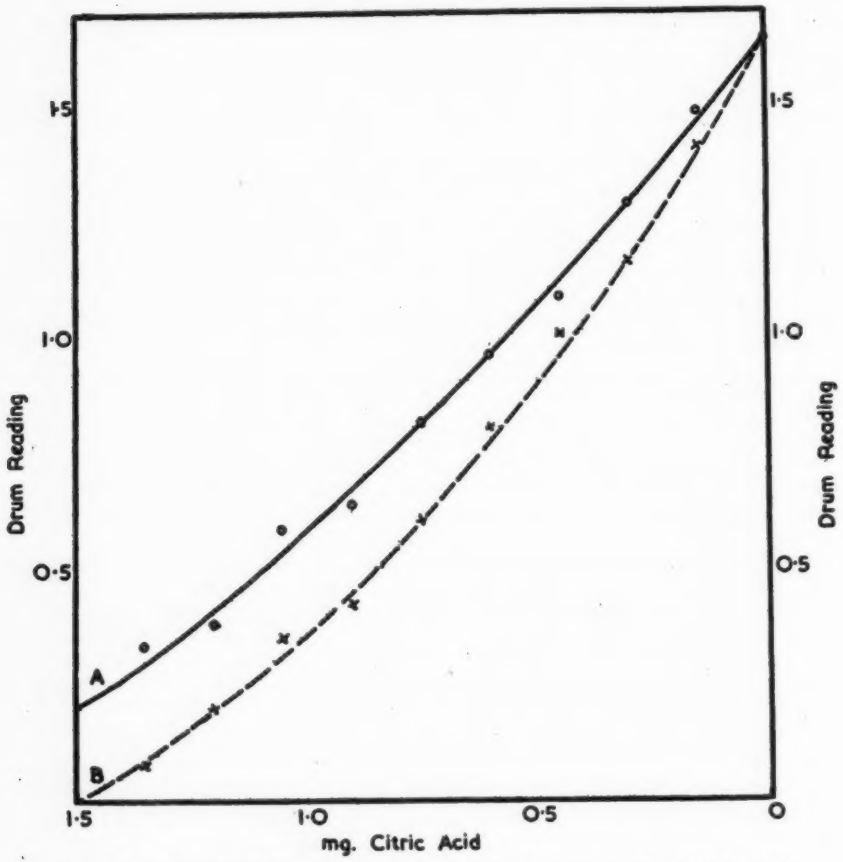
Glycerol has been used as a colour stabilizer, as recommended by Josephson and Forssberg[10], in preference to pyridine and dioxan, which have been used in the past. Experiments indicate that its use imparts a fairly high degree of stability to the coloured solution.

Using the method described it was found possible to obtain a satisfactory calibration curve with citric acid. It is interesting to note that this curve does not correspond with one prepared using a *n*-hexane solution containing equivalent quantities of pentabromoacetone. This indicates that, under the conditions of the experiment, the oxidation of citric acid to pentabromoacetone is not quantitative. However, the amount of pentabromoacetone produced appears to be constant for like amounts of citric acid, and the results obtained using the procedure described are reproducible.

It is important that the whole process should be carried out within the minimum of time, as unsatisfactory results have been obtained when it has been delayed.

The following table gives the results obtained from the addition of small quantities of citric acid to a solution of citric acid fermentation medium:—

Citric acid present mg.	*Citric acid added mg.*	*Total citric acid found mg.*	*Citric acid recovered mg.*
0·75	0·1	0·86	0·11
0·75	0·1	0·87	0·12
1·50	0·2	1·73	0·23
1·50	0·2	1·70	0·20



A.—Curve obtained with Spekker absorptiometer for known quantities of citric acid using the method outlined.

B.—The position which would be occupied by the curve if oxidation to pentabromoacetone were quantitative.

The medium used for the above analysis was found to contain 37·5 mg. citric acid per ml. using the method of analysis outlined above. By the Pucher, Vickery and Leavenworth volumetric method the same medium was found to contain 37·3 mg. citric acid per ml.

Interfering Substances

Krebs and Eggleston[7] have investigated, rather fully, the substances which interfere with the estimation of citric acid by its conversion to pentabromoacetone, and other workers, already referred to, have reported on interfering substances, though not so thoroughly.

Bromination has been recommended for the elimination of some interfering substances. This process is not favoured by the author as under certain conditions citric acid is oxidized to pentabromoacetone by bromine, in which case low results will be obtained in the estimation of citric acid. Boiling of the solution for analysis after slight acidification is also recommended for the destruction of interfering substances. This process is quite satisfactory and may be introduced as a preliminary treatment in the analysis of molasses fermentation media. Breusch and Tullus[11] propose an additional boiling with alkali. These authors have investigated the interference of thirty-four different substances in the colorimetric and argentometric estimation of citric acid. It is evident from their results that boiling with alkali is normally necessary only when citric acid is to be estimated argentometrically.

Breusch and Tullus[11] have found an appreciable interference by itaconic acid (a colour reaction equivalent to 0·23 mg. citric acid for 1 mg. itaconic acid), this substance being apparently unaffected by acid and alkaline boiling. However, it is unlikely that itaconic acid is produced in any appreciable amount in the fermentation of molasses solutions by *A. niger*.

From an examination of the work of Krebs and Eggleston, it appears that β-hydroxybutyric acid is the only substance which forms an appreciable amount of a bromination product, under the conditions of analysis for citric acid. As this substance might conceivably be produced in the fermentation of molasses, a sample was prepared by the method of Wislicenus[12]. When submitted to the scheme of analysis outlined above, 100 mg. of β-hydroxybutyric acid gave a colour reaction equivalent to 0·35 mg. citric acid. Such interference may be neglected. Pucher, Sherman and Vickery[5] have reported similar results.

National Chemical Research Laboratory,
South African Council for Scientific and Industrial Research,
Pretoria.

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REFERENCES

[1] Stahre, L., *Z. anal. Chem.*, 1897, **36**, 195.
[2] Wohlk, *Z. anal. Chem.*, 1902, **41**, 77.
[3] Pucher, G. W., Vickery, H. B., and Leavenworth, C. S., *Ind. Eng. Chem.* (Anal. Ed.), 1934, **6**, 190.
[4] Kretov, A. E., Panchenko, A. N., and Savich, K. K., *J. Gen. Chem.* (U.S.S.R.), 1931, **1**, 419.
[5] Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, 1936, **113**, 325.
[6] Perlman, D., Lardy, H. A., and Johnson, M. J., *Ind. Eng. Chem.* (Anal. Ed.), 1934, **16**, 515.
[7] Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, 1944, **38**, 426.
[8] Goldberg, A. S., and Bernheim, A. R., *J. Biol. Chem.*, 1947, **158**, 33.
[9] Taussky, H. H., and Shorr, E., *J. Biol. Chem.*, 1947, **169**, 103.
[10] Josephson, B., and Forrsberg, N., *J. Lab. Clin. Med.*, 1941, **27**, 267.
[11] Breusch, F. L., and Tullus, R., *Biochem. et Biophys. Acta*, 1947, **1**, 77 – 82
[12] Wislicenus, J., *Annalen*, 1869, **149**, 207.

CANE MOLASSES FERMENTATIONS

PART V. THE INFLUENCE OF INORGANIC ANIONS, PARTICULARLY CHLORIDE AND SULPHATE, UPON THE CITRIC-OXALIC ACID FERMENTATION

by

A. E. OXFORD, H. H. SEPHTON and G. C. A. VAN DER WESTHUIZEN

OPSOMMING

1. Die aanwesigheid van die chloriede van magnesium, kalium en natrium in betreklike hoë konsentrasies van half-normaal, in die gistingsbodem, onderdruk die vorming van oksaalsuur tot 'n veel groter mate as die van sitroensuur wanneer *A. niger* suiker na sitroensuur fermenteer.
2. Hierdie uitwerking is waarneembaar in beide die teenwoordigheid en afwesigheid van organiese stikstof in die gistingsbodem.
3. Die ooreenkomstige sulfate het oor die algemeen geen onderdrukkende invloed op die vorming van oksaalsuur nie.
4. Die uitwerking van nitrate is minder merkbaar as die van chloriedes.
5. 'n Verklaring van die bogenoemde feite, gebaseer op die invloed van die soute soos waargeneem op die pH van verdunde sitroensuuroplossings, word aan die hand gegee.
6. Die toepaslikheid van hierdie feite op die gisting van melasse met 'n hoë as-inhoud word aangedui.

SUMMARY

1. The chlorides of magnesium, potassium and sodium, when present in the medium in relatively high concentration of about half-normal, suppress the formation of oxalic acid to a very much greater degree than that of citric acid in the *Aspergillus niger* fermentation of sugar.
2. This effect is observable both in presence and absence of organic nitrogen in the medium.
3. The corresponding sulphates have on the whole no suppressant effect upon oxalic acid formation.
4. The effect of nitrate is less marked than that of chloride.
5. An explanation of the above facts is suggested based on the observed effects of these salts upon the pH of dilute citric acid solutions.
6. The relevance of these facts to the fermentation of cane molasses of high ash content is noted.

It is well known that oxalic acid is often produced in appreciable yield in the characteristic citric acid fermentation of sugar by *Aspergillus niger*. However, there is no generally accepted chemical mechanism yet proposed to account for the formation of either acid [*see* Birkinshaw[1]; Prescott and Dunn[2]], nor does there seem to be any mention in the literature of a sure method of entirely preventing the production of the undesirable oxalic acid without greatly affecting the yield of citric acid. The well-known recipe of Currie[3], *viz.*, high initial sugar, low initial inorganic salts and pH, undoubtedly often encourages the formation of citric acid and depresses that of oxalic acid, but in our experience (described below) a surprisingly large proportion of good citric acid-producing strains will also yield detectable amounts of oxalic acid under Currie's conditions. This observation has led us to re-examine the validity of one of Currie's requirements, *viz.*, low inorganic salt concentration, not only on account of the theoretical interest involved but also for a practical reason, since we are engaged in a study of the citric fermentation of Natal mill molasses, the ash content of which may be as high as 12 per cent, *i.e.*, about one-quarter of its fermentable sugar content.

The inorganic salts present in cane molasses consist largely of the sulphates and chlorides of magnesium, calcium, potassium and sodium. Very little phosphate or nitrate is present [*see* Spencer[4]]. Most or all of the calcium is precipitated as sulphate when the mash is made, but the other salts remain in solution, sometimes in a combined concentration of 3 – 4 per cent, to influence the fermentation. When fermenting cane molasses directly it is also not possible to fulfill another of Currie's conditions,

viz., low initial pH, because the molasses is so well buffered by amino-acids, etc., that an impracticably large amount of hydrochloric acid is required to bring the pH to within the range 2·5 – 3·5. A study has therefore been made of the effect of the sulphates and chlorides of magnesium, potassium and sodium at relatively high concentrations, upon the yield of oxalic acid in the citric fermentation of sugar, both in absence and presence of organic nitrogen, starting at pH 5–6, the usual pH of a mash made from Natal mill molasses. In order that any conclusions arrived at may have a general application, a large number of strains of black Aspergilli has been employed in this investigation.

EXPERIMENTAL

Cultures

The following list indicates the strains of black Aspergilli used for the main experiments and their source:—

Strain No.	*Origin*
I ..	A.T.C.C. 1015.
II ..	Prof. P. Simonart, Belgium: strain 1703.
III ..	Prof. P. Simonart, Belgium: strain 1739.
IV ..	Prof. P. Simonart, Belgium: strain 1734.
V ..	A.T.C.C. 6276.
VI ..	S.A.N.C.R.L. No. 293: contaminant on malt agar.
VII ..	Prof. P. Simonart, Belgium: strain 1711.
VIII ..	S.A.N.C.R.L. No. 456. Isolated from garden soil.
IX ..	S.A.N.C.R.L. No. 457. Isolated from garden soil.
X ..	L.S.H.T.M. No. A.79. Received originally from Prof. W. H. Peterson, Wisconsin, U.S.A.
XI ..	*A. pulverulentus*. L.S.H.T.M. No. Ac.12.
XII ..	Prof. P. Simonart, Belgium: strain 1695.
XIII ..	Prof. P. Simonart, Belgium: strain 1743.
XIV ..	L.S.H.T.M. No. BB1.

Key: A.T.C.C.—American Type Culture Collection.
L.S.H.T.M.—London School of Hygiene and Tropical Medicine.
S.A.N.C.R.L.—South African National Chemical Research Laboratory.

Cultural conditions

The fermentations were carried out in boiling tubes (15 × 2·5 cm.), each holding approximately 15 ml. of liquid before sterilization, which was by steaming. Incubation was at 20°C. – 25°C. for 16 – 21 days. A heavy inoculation was used to ensure quick formation of a complete felt.

Media

The basal medium was always that of Currie, but with a higher initial pH (5 – 6) to encourage oxalic acid formation, and to correspond roughly with that of a solution of a representative Natal mill molasses. The following were used for the experiments summarized in Tables 1 – 6 inclusive.

Medium A. Sucrose, 12·5 per cent; K_2HPO_4, 0·1 per cent; NH_4NO_3, 0·2 per cent; $MgSO_4.7H_2O$, 0·025 per cent. The pH was adjusted by means of phosphoric acid.

Medium B. As *A*, save that the sucrose concentration was only 9 per cent and the phosphate concentration was doubled. Being well buffered and of high initial pH, this medium is particularly favourable for oxalic acid production.

Medium C. A 25 per cent solution of Natal mill molasses was completely de-ionized by passing through columns first of the cation-exchange resin Amberlite IR-100H and then through the acid-absorbent resin IR-4B. It then gave no test for calcium, chloride or sulphate and only a trace of ash upon ignition. The following were then added: "Government" sugar (a slightly brown crystalline sugar, not of the highest refinement), 5 per cent; NH_4NO_3, 0·24 per cent; $MgSO_4.7H_2O$, 0·02 per cent; K_3PO_4, 0·06 per cent. The fermentable sugar content of this medium was about 15 per cent, and its pH was 5.

Methods

After the prescribed incubation period the felt was drawn up to the top of the tube and before removal was squeezed with a spatula, the tube being in an inclined position to remove as much adherent medium as possible. The total metabolic liquid was then filtered without suction through a No. 1 paper. Its citric acid content was determined by a modification of the well-known colorimetric method, based on the reaction between pentabromoacetone and sodium sulphide [*cf.* Perlman, Lardy and Johnson[5]], which has been described in Part IV of this series. Oxalic acid was determined by the usual $KMnO_4$-oxidation method after precipitation as the calcium salt. Reference to the tables will show that whenever a citric acid determination was carried out, the value obtained accounted for the greater part, and often almost the whole, of the net titratable acidity of the metabolic liquid, after allowing for the initial titratable acidity of the medium itself and the acidity due to oxalic acid. It may be assumed, therefore, that this holds also when no direct citric acid determination was carried out. Titratable acidity was determined by titration of 1·0 or 2·0 ml. of medium or metabolic liquid with 0·04N–NaOH phenolphthalein being used as indicator. With the molasses medium it was necessary first to dilute with distilled water (50 ml.) to lessen the colour, and to use a considerable quantity of indicator. In no case was the endpoint sufficiently obscured to indicate that gluconolactone was present in quantity. It was not thought necessary in this preliminary survey, which has been confined to the oxalic acid : citric ratio in the products, to determine sugar used up by fermentation and the non-acidic fermentation products like carbon dioxide and alcohol [*cf.* Birkinshaw, Charles, Raistrick and Stoyle[6]]. If a more detailed survey of the effect of any particular salt is to be made in the future, such determinations will, of course, have to be included, as well as that of carbon mycelium.

TABLE I

Citric and oxalic acid formation in Currie's medium (at pH 2 – 3) by a preformed felt in presence and absence o nitrate and phosphate.

Strain I. The medium contained also K_2SO_4 (0·02 per cent) to ensure the presence of K.[+]

Initial pH	*NH_4NO_3 per cent*	*KH_2PO_4 per cent*	*Net titratable acidity as citric acid per cent*	*Citric acid determind colorimetrically per cent*	*Oxalic acid (qualitatively)*
2·2	None	None	2·7	2·6	+
2·7	None	0·075	3·7	3·2	+ +
2·7	0·2	0·075	3·2	3·1	0

RESULTS

Oxalic acid production by the growth of numerous strains of *A. niger* on Currie's medium at initial pH 2·6

(*N.B.*—The pH of the medium was in this instance adjusted with HCl, not H_3PO_4.)

Of twenty-five different strains of *A. niger* derived from many different sources, all but 3 gave titratable acidities corresponding to >1 per cent of citric acid in the solution after 21 days' incubation, and of these 22 good citric-acid producers no less than 9 (*i.e.*, 40 per cent) yielded oxalic acid as well, as judged by the usual qualitative test, under these conditions. As a result of these tests, strains II, V, VI, VII and XII were chosen, among others, for the succeeding experiments because they gave particularly

TABLE II

Effect of magnesium chloride and magnesium sulphate upon oxalic and citric acid production from sucrose as sole source of carbon (Medium A).
Initial pH of medium = 5.

Strain of A. niger No.	*Concentration in medium of*		*Concentration of organic acids in metabolic liquid*			*Yield of oxalic acid as per cent of citric acid*	*Per cent titratable acidity due to oxalic acid*
	Additional mg. salt per cent	*Additional anion*	A *Net titratable acidity as citric acid* per cent	B *Citric acid determined colorimetrically* per cent	C *Oxalic acid* per cent	$=100\,C/B$	$=140\,C/A$
I	0	0	4·3	3·65	0·08	2·2	2·6
II	0	0	1·7	1·1	0·06	5·5	4·9
III	0	0	1·7	1·1	0·16	14·5	13·2
IV	0	0	3·2	—	0·0	0·0	0·0
I	3·5	0·34 N–Cl	2·5	2·25	0·01	0·4	0·6
I	7·0	0·68 N–Cl	1·9	2·0	0·0	0·0	0·0
II	3·5	0·34 N–Cl	0·2	—	0·01	—	7·0
II	7·0	0·68 N–Cl	0·2	0·3	0·0	0·0	0·0
III	7·0	0·68 N–Cl	0·95	0·8	0·01	1·2	1·5
IV	3·5 and 7·0	0·34 N and 0·68 N–Cl	0·0	0·0	0·0	—	—
I	3·5	0·29 N–SO_4	4·2	3·7	0·17	4·6	5·7
I	7·0	0·58 N–SO_4	4·1	3·7	0·30	8·1	10·2
II	3·5	0·29 N–SO_4	1·2	0·9	0·02	2·2	2·3
II	7·0	0·58 N–SO_4	0·9	0·7	0·0	0·0	0·0
III	7·0	0·58 N–SO_4	2·3	2·2	0·0	0·0	0·0
IV	3·5	0·29 N–SO_4	1·3	0·9	0·05	5·6	5·4
IV	7·0	0·58 N–SO_4	0·9	0·65	0·20	30·8	31·1
			Summary (average values).				
4*	0	0	2·7	—	0·075	—	3·9
7*	3·5 and 7·0	0·3 N–Cl	0·8	—	0·004	—	0·7
7*	3·5 and 7·0	0·25 N–SO_4	2·1	—	0·11	—	7·3

* Number of cultures tested as indicated above.

good reactions (II especially) for oxalic acid. If the pre-formed felt from a strain (*e.g.*, I) on Currie's liquid medium, which did not yield oxalic acid in the straightforward fermentation at pH 2·6, was transferred aseptically to fresh medium at an equally low pH, it could be induced to form oxalic acid as well as citric acid by omitting nitrate, or nitrate and phosphate from the medium (*see* Table I). It was concluded as a result of these preliminary experiments (and the conclusion was confirmed by much subsequent experience) that although certain strains are much more prone to yield oxalic acid than others, even at a low initial pH, yet no strain can be guaranteed not to produce oxalic acid, even in the simplest medium of low initial pH.

TABLE III

Effect of potassium chloride and of potassium nitrate upon oxalic and citric acid production from sucrose as sole source of carbon (Medium A).

Initial pH of medium = 6.

Strain of A. niger	*Additional salt added*	*Concentration in medium of*		*Concentration of organic acids in metabolic liquid*			*Yield of oxalic acid as per cent of citric acid*	*Per cent titratable acidity due to oxalic acid*
		Additional salt per cent	*Additional anion*	A *Net titratable acidity as citric acid per cent*	B *Citric acid determined colorimetrically per cent*	C *Oxalic acid per cent*	$=100\,C/B$	$=140\,C/A$
VI	0	—	—	2·75	2·75	0·12	4·4	6·1
XII	0	—	—	5·3	4·95	0·54	10·9	14·3
XIII	0	—	—	1·2	0·4	0·06	15·8	7·0
XIV	0	—	—	0·9	0·25	0·28	112·0	43·6
VI	KCl	3·0	0·35 N–Cl	0·7	0·75	0·02	2·1	4·0
VI	KCl	5·0	0·66 N–Cl	0·7	0·7	0·01	1·5	2·0
XII	KCl	3·0	0·35 N–Cl	1·7	1·9	0·07	3·7	5·8
XII	KCl	5·0	0·66 N–Cl	2·8	2·8	0·01	0·4	0·5
XIII	KCl	3·0	0·35 N–Cl	0·2	0·2	0·02	10·0	14·0
XIII	KCl	5·0	0·66 N–Cl	0·4	0·4	0·02	5·0	7·0
XIV	KCl	3·0	0·35 N–Cl	0·4	0·3	0·01	3·3	3·5
XIV	KCl	5·0	0·66 N–Cl	0·4	0·2	0·02	8·7	7·0
VI	KNO_3	3·0	0·3 N–NO_3	1·3	1·3	0·15	11·5	16·2
XII	KNO_3	3·0	0·3 N–NO_3	2·4	2·4	0·01	4·2	5·8
XIII	KNO_3	3·0	0·3 N–NO_3	0·4	0·55	0·10	18·2	35·0
XIV	KNO_3	3·0	0·3 N–NO_3	1·2	1·2	0·07	5·8	8·2
Summary (average values).								
4*	0	—	—	2·5	2·1	0·25	11·9	14·0
8*	KCl	—	0·35 N–Cl	0·9	0·9	0·02	2·2	3·1
4*	KNO_3	3·0	0·3 N–NO_3	1·2	1·2	0·07	5·8	8·2

* Number of cultures tested as indicated above.

Effect of certain "neutral" inorganic salts upon the citric-oxalic fermentation

For the reasons stated in the introduction, the chlorides and sulphates of magnesium, sodium and potassium were particularly studied. Potassium nitrate was also included in the experiment (*see* Table III), although in this instance there is a complication due to the possibility of the destruction of most of the anion during the course of growth

TABLE IV

Effect of sodium chloride and of sulphates upon oxalic and citric acid production in a medium (B) favourable to oxalic acid production.

Initial pH of medium = 6.

Strain of A. niger	*Additional salt added*	*Concentration in medium of*		*Concentration of organic acids in metabolic liquid*			*Yield of oxalic acid as per cent of citric acid*	*Per cent titratable acidity due to oxalic acid*
		Additional salt per cent	*Additional anion*	A *Net titratable acidity as citric acid per cent*	B *Citric acid determined colorimetrically per cent*	C *Oxalic acid per cent*	$=100\,C/B$	$=140\,C/A$
I	0	—	—	2·5	2·0	0·33	16·5	18·5
VI	0	—	—	1·0	0·7	0·14	20·0	20·0
XI	0	—	—	2·1	0·95	1·04	110·6	71·2
XII	0	—	—	4·7	4·0	0·58	14·5	17·3
I	NaCl	2·0	0·34 N–Cl	1·8	1·5	0·29	19·3	22·6
I	NaCl	4·0	0·68 N–Cl	1·1	1·0	0·06	6·0	7·6
VI	NaCl	2·0	0·34 N–Cl	1·0	1·0	0·01	1·0	1·4
VI	NaCl	4·0	0·68 N–Cl	0·7	0·55	0·01	1·8	2·0
XI	NaCl	2·0	0·34 N–Cl	0·75	0·5	0·19	38·0	35·5
XI	NaCl	4·0	0·68 N–Cl	0·6	0·3	0·10	33·3	23·3
XII	NaCl	2·0	0·34 N–Cl	1·4	1·35	0·27	20·0	27·0
XII	NaCl	4·0	0·68 N–Cl	1·3	1·3	0·04	3·1	4·3
I	Na_2SO_4	4·0	0·56 N–SO_4	1·2	0·8	0·0	0·0	0·0
VI	Na_2SO_4	4·0	0·56 N–SO_4	0·7	0·75	0·0	0·0	0·0
XI	Na_2SO_4	4·0	0·56 N–SO_4	0·5	0·0	0·28	0·0	78·4
XII	Na_2SO_4	4·0	0·56 N–SO_4	0·9	0·8	0·11	13·7	17·1
I	K_2SO_4	5·0	0·58 N–SO_4	0·25	0·25	0·0	0·0	0·0
VI	K_2SO_4	5·0	0·58 N–SO_4	0·5	0·5	0·0	0·0	0·0
XI	K_2SO_4	5·0	0·58 N–SO_4	1·5	0·4	0·84	210·0	78·4
XII	K_2SO_4	5·0	0·58 N–SO_4	0·6	0·4	0·06	15·0	14·0
				Summary (average values).				
4*	0	—	—	2·6	1·9	0·52	27·4	28·0
4*	NaCl	2·0	0·34 N–Cl	1·25	1·1	0·19	17·3	21·3
4*	NaCl	4·0	0·68 N–Cl	0·9	0·8	0·05	6·2	7·8
8*	Na_2SO_4 and K_2SO_4	— —	ca. 0·6 N–SO_4	0·8	0·5	0·16	32·0	28·0

* Number of cultures tested as indicated above.

TABLE V

Effect of the chlorides and sulphates of sodium and potassium upon the yield of oxalic acid from de-ionized molasses (Medium C) containing organic sources of nitrogen.
Initial pH of medium = 5.

Strain of 'A. niger *No.*	*Additional salt added*	*Concentration in medium of*		*Concentration of organic acids in metabolic acid*		*Per cent titratable acidity due to oxalic acid*
		Additional salt per cent	*Additional anion*	A *Net titratable acidity as citric acid per cent*	C *Oxalic acid per cent*	$=140\,C/A$
II	None	0	0	2·0	0·35	24·5
V	None	0	0	2·5	0·15	8·4
VI	None	0	0	2·2	0·15	9·5
II	NaCl	1·3	0·22 N–Cl	1·75	0·20	16·0
V	NaCl	1·3	0·22 N–Cl	1·7	0·03	2·5
VI	NaCl	1·3	0·22 N–Cl	1·1	0·0	0·0
II	NaCl	2·6	0·44 N–Cl	1·6	0·12	10·5
V	NaCl	2·6	0·44 N–Cl	1·65	0·02	1·7
VI	NaCl	2·6	0·44 N–Cl	1·85	0·01	0·8
II	NaCl	3·9	0·66 N–Cl	1·45	0·0	0·0
V	NaCl	3·9	0·66 N–Cl	1·05	0·01	1·3
VI	NaCl	3·9	0·66 N–Cl	1·35	0·02	2·1
II	KCl	1·6	0·21 N–Cl	1·85	0·15	11·3
V	KCl	1·6	0·21 N–Cl	1·9	0·11	8·1
VI	KCl	1·6	0·21 N–Cl	1·6	0·05	4·4
II	KCl	3·2	0·42 N–Cl	1·7	0·10	8·2
V	KCl	3·2	0·42 N–Cl	0·95	0·0	0·0
VI	KCl	3·2	0·42 N–Cl	1·8	0·01	0·8
II	KCl	4·8	0·63 N–Cl	1·3	0·04	4·3
V	KCl	4·8	0·63 N–Cl	0·8	0·0	0·0
VI	KCl	4·8	0·63 N–Cl	1·45	0·0	0·0
VI	Na_2SO_4	3·0	0·42 N–SO_4	2·3	0·24	14·6
II	Na_2SO_4	4·5	0·63 N–SO_4	1·8	0·37	28·8
V	Na_2SO_4	4·5	0·63 N–SO_4	2·65	0·42	22·2
VI	Na_2SO_4	4·5	0·63 N–SO_4	2·3	0·25	15·2
II	K_2SO_4	1·75	0·20 N–SO_4	2·2	0·97	60·8
II	K_2SO_4	3·5	0·40 N–SO_4	2·4	0·67	39·1
II	K_2SO_4	5·2	0·60 N–SO_4	2·2	0·55	35·0
V	K_2SO_4	5·2	0·60 N–SO_4	2·2	0·45	28·6
VI	K_2SO_4	5·2	0·60 N–SO_4	2·95	0·45	21·4

Summary (average values).

3*	None	0	0	2·2	0·22	14·0
18*	NaCl or KCl	—	0·2 N–Cl	1·5	0·05	4·7
9*	Na_2SO_4 or K_2SO_4	—	0·2 N–SO_4	2·3	0·49	29·8

* Number of cultures tested as indicated above.

TABLE VI

Effect of sodium chloride at low and high concentrations upon the yield of oxalic acid from de-ionized molasses (Medium C) containing organic sources of nitrogen.
Initial pH of medium = 5·5.

Strain of A. niger *No.*	*Additional NaCl per cent*	*Additional chloride ion*	*Concentration of organic acids in metabolic liquid* A *Net titratable acidity as citric acid per cent*	C *Oxalic acid per cent*	*Per cent titratable acidity due to oxalic acid* $=140\,C/A$
I	0	0	3·2	0·0	0·0
VII	0	0	1·5	0·0	0·0
VI	0	0	2·0	0·03	2·1
VIII	0	0	2·35	0·10	6·0
IX	0	0	0·85	0·06	9·9
I	0·05	0·009 N	3·2	0·08	3·5
VII	0·05	0·009 N	1·75	0·02	1·6
VI	0·05	0·009 N	2·7	0·07	3·6
VIII	0·05	0·009 N	1·85	0·13	9·8
IX	0·05	0·009 N	0·9	0·01	1·6
I	0·5	0·09 N	3·25	0·17	7·3
VII	0·5	0·09 N	1·6	0·0	0·0
VI	0·5	0·09 N	2·05	0·0	0·0
VIII	0·5	0·09 N	1·85	0·07	5·3
IX	0·5	0·09 N	0·95	0·06	8·8
I	4·0	0·68 N	2·25	0·0	0·0
VII	4·0	0·68 N	2·4	0·0	0·0
VI	4·0	0·68 N	0·5	0·0	0·0
VIII	4·0	0·68 N	1·6	0·0	0·0
IX	4·0	0·68 N	1·15	0·0	0·0
X*	0	0	1·6 (1·3 per cent citric acid found colorimetrically)	0·02	1·7
XI*	0	0	0·95 (0·6 per cent citric acid found colorimetrically)	0·13	19·2

* Medium consisted of de-ionized molasses only with no inorganic salts of any description added. These results are not included in the summary below.

Summary (average values).

5†	0	0	2·0	0·04	2·8
10†	0·5	0·1 N–Cl	2·0	0·06	4·2
5†	4·0	0·68 N–Cl	1·6	0·0	0·0

† Number of cultures tested.

and fermentation, either by reduction to ammonia or by reason of the incorporation of its nitrogen into the mycelium as protein, purines, etc.

The results are summarized in Tables II – VI inclusive, each table representing a separate experiment in which all the fermentation tubes were incubated side by side.

The results summarized in Table VII show the effect of relatively high concentrations of "neutral" inorganic salts upon the pH of citric acid solutions of concentrations likely to be met with in this fermentation. The alteration in pH is certainly not negligible in a number of instances. The significance of these observations is dealt with in the discussion which follows.

TABLE VII

Effect of high concentrations of inorganic salts upon the pH of citric acid solutions, of approximate pH 2. (Determined by means of a commercial pH meter.)

Concentration of			*Lowering (−) or raising (+) of pH due to inorganic salt*
Citric acid per cent	*Inorganic salt per cent*		
3	NaCl	4·4	−0·15
1·5	NaCl	4·0	−0·15
1·5	NaCl	0·5	0·0
1·5	KCl	5·0	−0·15
1·5	$MgCl_2$. $6H_2O$	7·0	−0·2
1·5	KNO_3	4·0	−0·05
1·5	Na_2SO_4	4·4	+0·3
1·5	K_2SO_4	5·0	+0·3
1·5	$MgSO_4$. $7H_2O$	7·0	+0·15

DISCUSSION

Inspection of Tables II – VI inclusive, particularly of the summaries of results at the foot of each, will show that if sufficient chloride ion is added to the medium, the formation of oxalic acid can be entirely or almost entirely suppressed with every black Aspergillus tested, no matter whether organic nitrogen is present in the medium or not. Although the yield of citric acid in a given incubation period is also often much diminished, there is always a marked decrease in the oxalic acid : citric acid ratio and in the percentage of fermentation acids accounted for by oxalic acid. The preferential suppression of oxalic acid formation by chloride ions, therefore, is indisputable. It is noteworthy that the nature of the accompanying cation is of little account, since the chlorides of magnesium, potassium and sodium all give the same result. The concentration of chloride ion required to eliminate oxalic acid production almost completely depends on the initial pH and buffering capacity of the medium. A comparison of the effects of NaCl presented in Tables IV and VI will show that the lower the initial pH and phosphate concentration, the less chloride is required to suppress oxalic acid formation. For ordinary purposes a half-normal concentration will suffice to achieve this end.

Equivalent concentrations of the corresponding sulphates have on the average no depressant effect upon oxalic acid production (*see* Tables II, IV and V). Thus, although the yield of citric acid is often diminished, the oxalic acid : citric acid ratio in their presence remains as high as before, or even higher. The effect of nitrate seems to be intermediate between that of chloride and sulphate.

The explanation of these facts is in all probability a very simple one. If we accept the view often put forward in the past, particularly by Jacquot[7], and supported by much experimental evidence, namely, that the production of oxalic acid of smaller equivalent weight than citric acid, is a compensatory mechanism called into play if the pH of the medium tends to become too high, it follows that the presence of any unmetabolized constituent of the medium which lowers the pH of dilute citric acid solutions will tend to prevent the formation of oxalic acid. Conversely, if any constituent raises the pH of dilute citric acid solutions, the mould will tend to make abnormally large amounts of oxalic acid. Table VII shows that chlorides, in about half normal concentration, have a small but real effect in lowering the pH of dilute citric acid solutions, whereas sulphates raise the pH of such solutions. Indeed, the difference between the pH values for 1·5 per cent citric acid, containing on the one hand 4 per cent of NaCl and on the other 4·4 per cent of Na_2SO_4, is as much as 0·45 of a unit. Nitrate in equivalent concentrations has less effect in lowering the pH than chloride, so it would be expected that it would not depress oxalic acid formation so greatly as chloride, which is in accordance with the facts (*see* Table III).

The significance of these results in connection with the direct fermentation of cane molasses of high ash content is obvious. In particular, further work is required to determine whether, when occurring together as in molasses, the effects of chloride and sulphate on the citric fermentation tend to cancel each other out. The composition of the ash of the molasses is perhaps the most important factor in determining whether oxalic as well as citric acid will be formed in its fermentation.

We are greatly indebted to Professors H. Raistrick and P. Simonart for the gift of cultures.

National Chemical Research Laboratory,
South African Council for Scientific and Industrial Research,
Pretoria.

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REFERENCES

[1] Birkinshaw, J. H., *Biol. Rev.*, 1937, **12**, 357.
[2] Prescott, S. C., and Dunn, C. G., "Industrial Microbiology", McGraw-Hill Book Co. Inc., New York and London, 1940.
[3] Currie, J. N., *J. Biol. Chem.*, 1917, **31**, 15.
[4] Spencer, G. L., "Meade's Cane Sugar Handbook", 8th Ed., J. Wiley & Sons, New York, 1945.
[5] Perlman, D., Lardy, H. A., and Johnson, M. J., *Ind. Eng. Chem.* (Anal. Ed.), 1944, **16**, 515.
[6] Birkinshaw, J. H., Charles, J. H. V., Raistrick, H., and Stoyle, J. A. R., *Phil. Trans. Roy. Soc.*, 1931, **220B**, 27.
[7] Jacquot, R., *Ann. des Ferm.*, 1938, **4**, 284; *Compt. rend. Soc. Biol.*, **127**, 1431; **128**, 69.

CANE MOLASSES FERMENTATIONS

PART VI. SOME OBSERVATIONS ON THE YELLOW PIGMENTS PRODUCED BY CERTAIN STRAINS OF *ASPERGILLUS NIGER*

by

A. E. OXFORD

OPSOMMING

Die geel kleurstof wat deur sommige tipes van *A. niger* geproduseer word is oplosbaar in water en verskil van alle ander bekende geel kleurstowwe deur swamme geproduseer. Dit word met tolueen ge-ekstraheer uit die gedroogde swamweefsels van tipes wat die geel kleursel produseer en is dus maklik beskikbaar vir ondersoek. Daar is geen verband tussen die vermoë van *A. niger* tipes om hierdie geel kleurstof te produseer en hulle vermoë om suiker na sitroensuur om te sit nie.

SUMMARY

The water-soluble yellow colouring material produced by certain strains of *Aspergillus niger* is not identical with any of the known mould pigments of that colour. It is extracted by toluene from the dried mycelia of strains giving rise to yellow colorations and is thus readily available for detailed study. There is no correlation between the ability of *A. niger* strains to produce a yellow colour and their power of converting sugar to citric acid.

All workers who have handled a large number of cultures of *Aspergillus niger* and related species of black Aspergilli have become aware of the presence of a yellow or orange colour, as well as the invariable dark brown or black, in a surprisingly large proportion of the whole. Thus, Thom and Raper[1] state that "In the reverse or underside of the mycelium and in the culture substratum, a range of shades from yellow to reddish brown, and even very dark shades, is reported for particular species growing upon specified substrata. The chemical reactions back of these colours are not known for the black Aspergilli." The reason for this lack of knowledge concerning the *A. niger* pigments other than black is doubtless to be found in the difficulty of obtaining them free from the preponderant black pigment of the spores. It sometimes happens, however, that a yellow colour develops in the metabolic liquid in the case of old liquid cultures, and this has been ascribed to the production of flavins, even riboflavin [Lavollay and Laborey[2]; Knobloch and Sellmann[3]]. A similar instance has been observed in this series of investigations when an established felt was used to ferment a fresh batch of medium, which was intermittently stirred and circulated underneath it. However, there seems to be no positive and unequivocal identification of any *A. niger* pigment with a known substance and this work was undertaken in order to find out if the yellow pigment or pigments of the black Aspergilli did in fact include riboflavin or any other known fungal pigment. It was also desired to find out if there was any correlation between the tendency to produce yellow pigment and ability to form citric acid from sugar.

EXPERIMENTAL

Cultures

About 10 per cent of the 100 or more strains of black Aspergilli in our collection (*see* Part III) showed a yellow to orange reverse when cultivated on glucose-Czapek agar. Eight of these "yellow" strains were selected for further study, namely, Nos. 289, 304, 375, 378, 382, 479, SB, P1. The last two are recent isolates. For comparison, two strains which never exhibited any colour but black were also selected, *viz.*, Nos. 224 and 389.

Medium

Since the yellow colours were exhibited better on glucose-Czapek agar than on sucrose-Czapek agar, it was decided to grow the above moulds on liquid 5 per cent glucose-Czapek medium at pH 5·7 initially with KNO_3 (0·2 per cent) as sole source of nitrogen. Usually 250 ml. of medium was employed in a 750 ml. Erlenmeyer flask. Incubation was at 20°C. (room temperature) for at least 18 days.

RESULTS

1. Pigment in metabolic liquid

The yellow colour, if shown at all, invariably appeared first of all in the felt, usually on the underside. It diffused into the acid medium only during the later stages of incubation, and could then be extracted from the metabolic liquid by shaking with ether. In no instance did a yellow metabolic liquid contain riboflavin. This was proved by suitable additions of neutralized metabolic liquid (up to 1 ml.) to 5 ml. of the Snell and Strong[4] basal medium for the microbiological assay of riboflavin using *Lactobacillus casei*. Although controls containing authentic riboflavin always showed growth of the bacillus, the cultures containing the mould metabolic liquids never grew at all. The yellow metabolic liquids gave no colour with ferric chloride and were unchanged in colour by addition of sodium hydroxide or ammonium hydroxide.

2. Pigment in mycelium

The washed and vacuum-dried mycelium yielded its yellow pigment or pigments, unmixed with brown or black, to boiling toluene. This seemed to be the best solvent for the purpose; the others tried, either extracted no colour or dissolved the black pigment also. All the 8 "yellow" strains yielded yellow toluene extracts, whereas the two "non-yellow" strains (Nos. 224 and 389) gave colourless toluene extracts. Riboflavin is quite insoluble in boiling toluene.

3. Properties of yellow toluene extracts of dried mycelium

In all cases save one, the yellow colour of the toluene extract remained practically unaltered both in shade and in intensity, on dilution with a little alcoholic ammonia. The exception was strain 479. Its yellow toluene extract was immediately and completely decolorized on addition of alcoholic ammonia. It is noteworthy that this mould, although undoubtedly *A. niger*, hardly formed any spores at all on the glucose-Czapek liquid medium. A pure yellow alcoholic extract of the dried mycelium, therefore, could be made and this also was decolorized by a drop of aqueous ammonia.

DISCUSSION

The reactions of the water-soluble yellow pigment show that it is not identical with any of the known crystalline mould pigments of that colour [*e.g.*, ravenelin, citrinin, flavoglaucin, auroglaucin, fulvic acid, citromycelin (*see* Gould[5] for details)] which give colours with ferric chloride, or a marked change of colour with sodium hydroxide. Nor is it riboflavin. It is quite possible that there are at least two yellow mycelial pigments soluble in toluene, only one of which is decolorized by alcoholic ammonia. It would be difficult to detect this pigment in the presence of the other and in only one instance was it extracted unmixed with the pigment which remains unaffected by ammonia and which is probably identical with the water-soluble pigment. Since these pigments are so easily extractable from the mycelium, their chemical study is not a matter of great difficulty, and will be undertaken.

There is clearly no correlation between ability to produce a yellow colour and the power of converting sugar into citric acid. Good and poor citric acid-producers are found both in the minority of strains which tend to yield the yellow reverse on solid media, and in the majority which do not.

National Chemical Research Laboratory,
South African Council for Scientific and Industrial Research,
Pretoria.

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REFERENCES

[1] Thom, C., and Raper, K. B., "A Manual of the Aspergilli", The Williams & Wilkins Co., Baltimore 1945, 236.
[2] Lavollay, J., and Laborey, F., *Compt. rend.*, 1939, **208**, 1056.
[3] Knobloch, H., and Sellmann, R., *Zentr. Bakt. Parasitenk.*, 1941, **103**, 277.
[4] Snell, E. E., and Strong, F. M., *Ind. Eng. Chem.* (Anal. Ed.), 1939, **11**, 346.
[5] Gould, B. S., "Chemical Compounds Formed From Sugar by Molds", Sugar Research Foundation Inc., New York, Sci. Rpt., Series No. 7, 1947.

THE ACTION OF ANHYDROUS ALUMINIUM CHLORIDE ON PHENYL CINNAMATE

by

J. D. M. SIMPSON and S. S. ISRAELSTAM

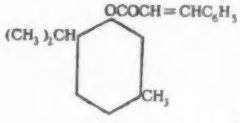
OPSOMMING

Fenielsinnamaat ondergaan die Fries-verandering wanneer dit met watervrye aluminiumchloried behandel word.

SUMMARY

Phenyl cinnamate undergoes the Fries rearrangement when it is treated with anhydrous aluminium chloride.

It has been shown by Rosenmund and Schnurr[1] that when the Fries rearrangement of a phenolic ester is carried out at low temperatures only the para-hydroxy ketone is formed, whereas at high temperatures (between 140°C. and 180°C.) the ortho-hydroxy ketone is the product. The same authors[2] showed that when thymyl cinnamate **I** in nitrobenzene, is treated with anhydrous aluminium chloride at room temperature 1-methyl-3-hydroxy-4-isopropyl styryl ketone **II** is formed.



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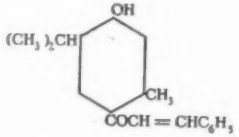
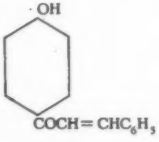
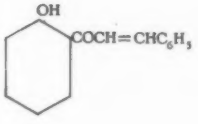


II

The action of anhydrous aluminium chloride on a nitrobenzene solution of phenyl cinnamate at room temperature was found to give the para-hydroxy compound *p*-hydroxyphenyl styryl ketone **III**. When phenyl cinnamate was heated together with anhydrous aluminium chloride at 180°C., the expected isomeric ketone **IV** was obtained.



III



IV

These styryl ketones, **III** and **IV**, have been obtained respectively by Kostanecki and Tambor[3] and by Kostanecki and Furenstein[4] by the action of benzaldehyde on the corresponding para- and ortho-hydroxyacetophenones.

EXPERIMENTAL

p-Hydroxyphenyl styryl ketone III

Finely-powdered anhydrous aluminium chloride (2 g.) was added to a solution of phenyl cinnamate (2·5 g.) in dry nitrobenzene (30 ml.) and the mixture allowed to stand at room temperature for forty-eight hours. The mixture was poured into ice-cold hydrochloric acid and the nitrobenzene was removed by steam distillation. After cinnamic acid (0·2 g.) had been extracted from the non-volatile residue the latter was treated with dilute sodium hydroxide solution. The alkaline solution on acidification yielded *p*-hydroxyphenyl styryl ketone (0·35 g., 17 per cent), which crystallized from dilute alcohol in yellow needles, m.p. 171°C. – 172°C. Kostanecki and Tambor[3] give m.p. 172°C. (Found C, 80·16; H, 5·32. $C_{15}H_{12}O_2$ requires C, 80·33; H, 5·40 per cent.) Phenyl cinnamate (1·6 g.) was recovered from the portion insoluble in sodium hydroxide.

o-Hydroxyphenyl styryl ketone IV

An intimate mixture of phenyl cinnamate (2·25 g.) and anhydrous aluminium chloride (2 g.) was placed in a test-tube fitted with a stopper carrying a calcium chloride tube, and a thermometer which reached almost to the bottom of the tube. The reaction mixture was heated in an oil bath, the temperature of which was slowly raised. At 80°C. a vigorous evolution of hydrogen chloride occurred, and the temperature of the mixture rose rapidly to 120°C. The temperature of the bath was raised until the temperature of the mixture was 180°C., and the reaction mixture was maintained at that temperature for one hour. The cooled melt was treated with ice and hydrochloric acid, when a resinous product was obtained. This yielded golden needles of *o*-hydroxyphenyl styryl ketone (0·9 g., 40 per cent), m.p. 87°C. – 88°C. Kostanecki and Furenstein[4] give m.p. 88°C. – 89°C. (Found C, 80·23; H, 5·46; $C_{15}H_{12}O_2$ requires C, 80·33; H, 5·40 per cent.)

Cinnamic acid (0·4 g.) and phenyl cinnamate (0·5 g.) were also recovered.

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Department of Chemistry and Chemical Engineering,
University of the Witwatersrand,
Johannesburg.

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REFERENCES

[1] Rosenmund and Schnurr, *Annalen*, 1928, **460**, 56.
[2] Rosenmund and Schnurr, *ibid.*, 80.
[3] Kostanecki and Tambor, *Ber.*, 1899, **32**, 1294.
[4] Kostanecki and Furenstein, *Ber.*, 1898, **31**, 1757.

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